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NPHS2 R229Q functional variant is associated with microalbuminuria in the general population

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***NPHS2* R229Q functional variant is associated with microalbuminuria in the general population.**

Background. Microalbuminuria is a risk factor for developing end-stage renal disease and cardiovascular events. Mutations in *NPHS2* have been shown to cause autosomal-recessive nephrotic syndrome. Recently, a functional polymorphism of this gene (R229Q) was described and associated with a maturity-onset form of nephrotic syndrome. We have investigated whether the carrier status of this novel genetic variant is associated with microalbuminuria in individuals from the general population.

Methods. Demographic, cardiovascular risk factors, and renal phenotypes in 1577 individuals from a cross-sectional–based study were collected following the general guidelines of the WHO-MONICA project (monitoring trends and determinants in cardiovascular diseases). Blood and urine samples were obtained. Microalbuminuria was determined using a semiquantitative protocol, and DNA was extracted from peripheral lymphocytes.

Results. A strong association was found between the 229Q allele and microalbuminuria ($P = 0.008$). The presence of the 229Q allele was still associated with a 2.77-fold increased risk of presenting microalbuminuria even after adjustment for age, ethnicity, hypertension, obesity, and diabetes in a multiple logistic regression model. In addition, a statistically significant interaction was identified between the presence of the 229Q allele and body mass index (BMI) ($P = 0.01$), suggesting an additive effect between the 229Q allele and other risk factors for microalbuminuria.

Conclusion. These data have important implications for the understanding of microalbuminuria in the general population and may contribute to better ways of disease prediction and prevention.

Key words: genetics, NPHS2, microalbuminuria.

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Podocin is a membrane protein member of the stomatin family. It is expressed in the podocytes and has an important role in the maintenance of the kidney glomerular capillary permselectivity. Its function is apparently exerted through interactions with other proteins, such as nephrin, also physiologically involved in the maintenance of podocyte function, namely the adequate function of the slit diaphragm membrane [1]. Podocin itself has been shown to localize to the slit diaphragm [2].

The gene expressing podocin, *NPHS2*, has recently been cloned. Mutations in *NPHS2* can cause nephrotic syndrome, transmitted in an autosomal-recessive manner, with a histopathologic appearance of focal and segmental glomerulosclerosis (FSGS) [3]. This type of nephrotic syndrome usually is diagnosed in preschool children, but a late-onset form has recently been described in patients who are compound heterozygotes, with one allele harboring a relatively common R229Q variant [4]. This 229Q variant, present in approximately 4% of Western populations, encodes a protein with lower affinity for binding to nephrin. However, there are no studies to date focusing on the late consequences of 229Q heterozygosity.

It has now been clearly demonstrated that microalbuminuria is a risk factor for developing end-stage renal disease (ESRD) and cardiovascular events. Microalbuminuria can also be considered a risk factor for developing diabetic nephropathy. In hypertensive patients, it is considered a marker of organ damage [5]. Nevertheless, little is known about the molecular determinants of this condition.

We have tested whether the R229Q functional polymorphism heterozygosity can contribute to the appearance of microalbuminuria in the general population. In order to answer this question we have studied the association of the *NPHS2* R229Q polymorphism with different renal phenotypes in a large, ethnic heterogeneous, urban population.

METHODS

Study population

A cross-sectional study of risk factors for cardiovascular diseases was performed in the urban population of Vitoria, Brazil, following the general guidelines of the WHO-MONICA project [6]. A sample of 2044 individuals (from an eligible population of 137,330) of both sexes, aged 25 to 64 years, was invited to participate in the study. The subjects were chosen after a random selection of domiciles. In each residence only one subject was invited according to the nearest birthday.

From this sample, 1577 attended the clinic visit and were evaluated for height, weight, smoking habits, blood pressure measurements, and use of medicines. Blood and urine samples for determination of plasma cardiovascular risk factors (blood glucose, total cholesterol, lipoprotein fractions, and triglycerides) were collected after a 12-hour fasting period. All measurements were performed according to standard techniques.

During the clinic visit all subjects were also submitted to a racial classification according to a validated questionnaire for the Brazilian population [7, 8]. Subjects were classified as European descent or African descent according to a set of phenotypic characteristics (skin color, hair texture, shape of the nose, aspect of the lip, and jaw position). On the basis of these characteristics, mulattos are considered racially mixed subjects.

Diabetes mellitus was defined as fasting glucose >125 mg/dL. Obesity was defined as a body mass index (BMI) above 30 kg/m^2. Overweight was defined as a BMI between 25 and 29.9 kg/m^2.

The study protocol was approved by the Institutional Review Board of the Espirito Santo Federal University, and all participants read and signed an approved informed consent.

Blood pressure phenotype determination

Blood pressure was measured by trained technicians using a standard mercury sphygmomanometer on the left arm after 5 minutes' rest with the subject in the sitting position. The first and fifth phase of Korotkoff sounds were used for systolic and diastolic blood pressure, respectively. Systolic and diastolic blood pressure was calculated from two readings taken by two different observers. The two measurements were obtained with a minimal interval of 10 minutes. Hypertension was defined as the mean systolic blood pressure of ≥140 mm Hg and/or diastolic blood pressure of ≥90 mm Hg [9]. Pulse pressure was the difference between systolic and diastolic blood pressures. Data on antihypertensive drug use (16.7% of individuals) were not included in the definition of hypertensive individuals because only 30% of these treated subjects had normal blood pressure. Conservatively, these were classified as normotensive, and the remaining ones were included as hypertensive individuals.

Renal phenotype determination

We determined serum urea and creatinine by standard techniques. Creatinine clearance was calculated in all participants using the formula of Cockroft and Gault [10]. Calculated creatinine clearance was standardized to 1.73 m^2 of body surface area. Mild renal dysfunction was defined as a calculated creatinine clearance of 60 mL/min/1.73 m^2 or less [11]. Urine was collected from all patients overnight (7 p.m. to 7 a.m.) and stored at room temperature. A sample was separated the same day to determine albumin concentration by a semiquantitative assay using a commercial kit (Micral-Test II, Boehringer Mannheim, Mannheim, Germany) designed to detect albumin concentrations from 2 to 20 mg/dL. Individuals with albumin concentration higher than 2 mg/dL in urine were considered positive for microalbuminuria.

Assessment of *NPHS2* R229Q genotype

The 5-mL blood samples were drawn into tubes containing EDTA. Genomic DNA was extracted from peripheral leukocytes using standard techniques. *NPHS2* exon 5 was polymerase chain reaction (PCR)-amplified using the following primers: F 5′-AGGATTTACCAC AGGATTAAGTTGTGCA – 3′ and R 5′-TAGCTATG AGCTCCCAAAGGGATGG – 3′. Three microliters of unpurified PCR product were diluted to 10 μL in recommended restriction buffer containing 5 U of *ClaI* and digested at 37°C overnight. The PCR products were visualized by electrophoresis in a 3% agarose gel with ethidium bromide and stored in digital form.

Quality control for these assays was assessed by randomly selecting 50 samples to be regenotyped by two independent technicians.

Statistical analysis

Allele and genotype frequencies among study participants were analyzed with the Chi-square test and multivariate logistic regression using the statistical package StatView for Windows, version 5.0 (SAS Institute, Inc., Cary, NC, USA). Correction for multiple comparisons was not performed in any of the analyses in the present study. To test for differences in various characteristics, Student *t* test was used for continuous variables and the Chi-square test was used for categorical variables.

Hardy-Weinberg equilibrium for the distribution of genotypes was estimated by the Chi-square test. The odd ratios (OR) for different association models were calculated with 95% confidence interval (CI) and two-tailed *P* values.

Table 1. Population characteristics per genotype

	R229R	R229Q	*P* value
Number	1.491	85	
Age *years*	44.9 (10.9)	43.5 (10.7)	0.26
Male%	45.4	47.7	0.69
Systolic blood pressure *mm Hg*	127.99 (22.0)	127.21 (19.5)	0.75
Diastolic blood pressure *mm Hg*	84.21 (14.1)	84.16 (11.9)	0.97
Pulse pressure *mm Hg*	43.78 (13.8)	43.05 (13.2)	0.63
Hypertension%	33.7	37.2	0.51
BMI	26.30 (4.9)	26.02 (4.2)	0.61
WHR	0.88 (0.09)	0.88 (0.09)	0.57
Overweight%	36.9	39.5	0.84
Obesity%	19.7	16.3	0.51
Smoking status%	22.2	20.9	0.67
Cholesterol *mg/dL*	214.88 (48.1)	206.07 (40.6)	0.10
Triglycerides *mg/dL*	138.06 (128.8)	129.79 (111.2)	0.56
HDL-cholesterol *mg/dL*	45.30 (12.4)	46.47 (12.2)	0.40
LDL-cholesterol *mg/dL*	142.67 (39.9)	134.47 (33.5)	0.06
Glucose *mg/dL*	105.08 (32.1)	103.39 (30.9)	0.64
Diabetes%	8	4.7	0.27

Abbreviations are: BMI, body mass index; WHR, waist hip ratio. Data are presented as mean (standard deviation). Diabetes mellitus was defined as fasting blood glucose >125 mg/dL.

Table 2. Renal phenotypes and R229Q allele

	R229R	R229Q	*P* value
Serum urea *mg/dL*	27,34 (10,0)	27,85 (7,6)	0.64
Serum creatinine *mg/dL*	0,97 (0,2)	0,97 (0,2)	0.81
Creatinine clearance *mL/min*[a]	89,40 (25,6)	90,75 (24,6)	0.63
Mild renal dysfunciton%[b]	9.76	8.14	0.62
Microalbuminuria%	6.11	14.75	0.008

[a]Estimated by the formula of Cockroft and Gault.
[b]Estimated creatinine clearance <60mL/min.

Table 3. Logistic regression model for presence of proteinuria

Variable	OR	95% CI	*P* value
Diabetes	4.6	2.33–9.06	<0.0001
Overweight	2.99	1.51–5.93	0.002
Obesity	3.27	1.53–6.95	0.002
Hypertension	1.39	0.80–2.43	0.25
Age	0.98	0.95–1.00	0.09
Ethnicity	0.94	0.74–1.18	0.57
Presence of R229Q allele	2.77	1.21–6.34	0.02

Logistic regression analysis that allowed for age, sex, diabetes mellitus, ethnicity, obesity, and hypertension explored the association between genotype and risk of microalbuminuria.

P values less than 0.05 on a two-sided test were considered significant.

RESULTS

In the 1577 individuals investigated in this study we found 85 heterozygous individuals for the R229Q substitution and one individual homozygous for this allele. Allele frequency was 2.76%, which is in accordance with previous findings. Allele and genotype frequencies were in Hardy-Weinberg equilibrium ($P = 0.99$). Interestingly, a tendency toward an increased frequency of individuals harboring the 229Q allele was found in Caucasian individuals, as compared with both mulatto and black individuals (6.9% in the European descent group, 4.9% in the mulatto group, and 2.5% in the African descent group; $P = 0.18$). For all further analysis we excluded the 229Q homozygous individual and compared genotype-phenotype associations regarding heterozygosity for the R229Q variant.

Population characteristics per genotype are presented in Table 1. We were able to collect complete phenotypic information regarding microalbuminuria on 1027 individuals. There was no significant difference between the samples of individuals with and without urinary albumin excretion data regarding the main demographic predictors of microalbuminuria. No significant difference was present between any of the studied variables and the presence of the 229Q allele. Renal phenotypes per genotype are presented in Table 2. No significant difference regarding creatinine clearance and the presence of the 229Q allele was observed. Nevertheless, 9 out of 61 individuals (14.8%) with the 229Q allele presented with microalbuminuria, a significantly different figure from the one observed in individuals without the 229Q allele; only 59 out of 966 (6.1%) individuals presented a positive semiquantitative measurement of microalbuminuria ($P = 0.008$).

In order to better study the association between the presence of the 229Q allele and presence of albuminuria we have constructed multiple logistic regression models seeking variables associated with significant albuminuria in our sample. These data are presented in Table 3. The presence of the 229Q allele was still associated with a 2.77-fold increased risk of presenting microalbuminuria even after adjustment for age, ethnicity, hypertension, obesity, and diabetes.

In addition, a statistically significant interaction was identified between the presence of the 229Q allele and both BMI, as a continuous variable ($P = 0.01$), and obesity, as a dichotomous variable ($P = 0.008$), suggesting an additive effect between the 229Q allele and other risk factors for microalbuminuria (Fig. 1). Although the presence of the R229Q allele does not significantly increase the risk of microalbuminuria in non-obese individuals, it is associated with an increasingly higher risk of microalbuminuria in obese individuals.

DISCUSSION

ESRD is a major health concern. In the United States the total estimated cost for Medicare patients with ESRD in 1998 was $12.04 billion, and it is estimated that by 2010 there will be 651,330 long-term ESRD patients [12].

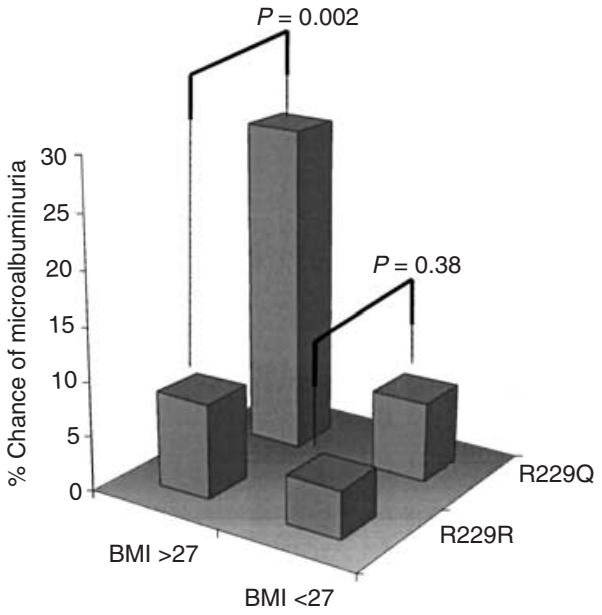

Fig. 1. Chance of microalbuminuria in percentage relative to R229Q genotype and a body mass index (BMI) >27. Groups were compared using the Chi-square test.

Although a number of primary kidney diseases may lead to ESRD, the majority of cases are secondary, namely due to diabetic and hypertensive nephropathies. Despite a number of proposed treatment and measures to delay progression to this condition, little is known regarding their molecular determinants of susceptibility.

Here we show for the first time that the presence of the 229Q allele of the *NPHS2* gene, recently identified as a cause of autosomal-recessive late-onset nephrotic syndrome and present with an allele frequency of 4% control populations, is associated with an increased risk of microalbuminuria in the general population. Presence of this allele was still significantly associated with a 2.77-fold increased risk of microalbuminuria even after adjustment for confounders such as age, blood pressure, obesity, diabetes, and ethnicity.

Several lines of evidence indicate that microalbuminuria is a valuable surrogate marker for both disease morbidity and mortality. In diabetes it has been shown that individuals presenting microalbuminuria have an enhanced risk of developing progressive renal failure compared with subjects with a normal albumin excretion [13]. It has been suggested that the same situation may also be operant in hypertension [14]. In addition, it has been shown that microalbuminuria enhances the risk for cardiovascular mortality in diabetic patients [13] and in patients with essential hypertension. Finally, it has recently been published that an increased albumin excretion is also associated with increased mortality even in the general population [15].

Even though microalbuminuria is considered a marker of renal and cardiovascular morbi-mortality, questions regarding a real causative role of proteinuria are still highly debatable. Either way, our findings should constitute highly useful information. If, on the one hand, microalbuminuria is really a cause of increased morbidity and progression to ESRD or cardiovascular mortality, then identification of individuals at increased risk of this condition can prove to be of major public health importance. If, on the other hand, microalbuminuria is only a confounding marker for increased disease mortality, then knowledge of genetic risk factors associated with a "benign microalbuminuria" should be incorporated into algorithms of risk stratification.

Besides showing an increased risk of microalbuminuria, our data also suggest that comorbidities such as obesity may further help to unmask the deleterious effect of this allele regarding microalbuminuria. This fact may suggest that physical factors such as blood flow may have an important role in the cascade of pathologic events leading to loss of podocyte homeostasis and proteinuria. It also may point toward important targets for disease prevention and individualization of renoprotective treatments.

Our study has some potential limitations. The methodology used for identifying individuals with microalbuminuria was based on a semiquantitative protocol. Although this approach may, at first, be considered less sensitive or less specific, our data show highly concordant figures when compared with studies that relied upon quantitative methods of microalbuminuria measurement [16]. In addition, important predictors of microalbuminuria, such as age, obesity, and diabetes, were also associated with this phenotype in our population, further validating it as a robust variable. Finally, the protocol used may also detect proteinuria above 200 mg/L and we cannot completely exclude the presence of individuals with macroalbuminuria in our sample. However, one would not expect a great number of individuals with macroalbuminuria in a sample randomly selected from the general population with no known renal disease.

CONCLUSION

A better understanding of the role of the R229Q functional variant regarding progression to ESRD and occurrence of cardiovascular events will not only shed light in unknown aspects of podocyte and glomerular physiology, but constitutes an important tool in disease prediction and prevention.

NOTE ADDED IN PROOF

Patent pending for the use of this gene variant in disease prevention, risk assessment, and renoprotective treatments.

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